

# Liriodenine, Early Antimicrobial Defence in *Annona diversifolia*

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Annonaceae aporphine alkaloids, of which liriodenine is the most abundant, have not been extensively studied from a biological standpoint. The goal of this study was to investigate the role of liriodenine in antimicrobial defense during early developmental stages in *Annona diversifolia*. The fungi *Rhizopus stolonifer* and *Aspergillus glaucus*, which are responsible for seed deterioration, were isolated during imbibition, and their antifungal activity was determined by diffusion, macrodilution, and metabolic inhibition assays using purified liriodenine and alkaloid extracts obtained from embryos, radicles, and roots at early developmental stages. The presence of liriodenine in extracts was quantified by high-performance liquid chromatography. Purified liriodenine and alkaloidal extracts inhibited both fungi, and there was a positive relationship between extract activity and amount of liriodenine contained therein. The quantity of liriodenine present in extracts suggests its importance in controlling other phytopathogens.

**Key words:** Alkaloid, Annonaceae, Early Development

## Introduction

Plants are not able to move in search of a more suitable environment for growth and development. As such, they are often subjected to environmental stress caused by biotic or abiotic factors. This fact is probably one of the reasons why plants have acquired and perfected over thousands of years of evolution defense mechanisms that allow them to respond to external attacks, and therefore live in very different environments (Dixon, 2001; Hartmann, 2007).

Damage caused to plants by invading pathogens (bacteria, fungi, viruses, and viroids) or by herbivory frequently leads to metabolic responses aimed at the survival of the species. Such responses require the allocation of energy resources to build up stores of defense substances, involving a complete biosynthetic machinery based on a process of differentiation (Luckner, 1980; Wink, 2010). For example, *Manduca sexta* herbivory of *Nicotiana* leaves and flowers increases the amount of alkaloids in new leaves and flower nectar (Baldwin, 1999; Adler *et al.*, 2006).

Liriodenine (Fig. 1) is a benzyloisoquinoline alkaloid (oxoaporphine) that has antibacterial (Hufford *et al.*, 1975, 1980; Villar *et al.*, 1987; Nissanka *et al.*, 2001; Rahman *et al.*, 2005; Wirasathien *et al.*, 2006), antifungal (Hufford *et al.*, 1980; Nissanka *et al.*, 2001; Rahman *et al.*, 2005), antiprotozoal (Waechter *et al.*, 1999; Wirasathien *et al.*, 2006), and cytotoxic (Chen *et al.*, 1997; De Siqueira *et al.*, 1998; Chang *et al.*, 2004; Wirasathien *et al.*, 2006) properties. To date, liriodenine has demonstrated activity against 22 phytopathogenic agents, mostly *in vitro* and seven *in situ* (Table I), which suggests

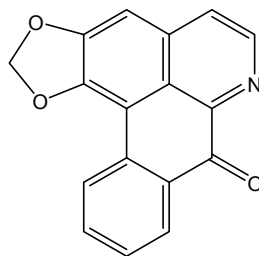


Fig. 1. Chemical structure of liriodenine.

Table I. Liriodenine activity on phytopathogenic organisms.

| Phytopathogen                        | Plant disease                |              | Sensibility |                 | Reference                     |
|--------------------------------------|------------------------------|--------------|-------------|-----------------|-------------------------------|
| <i>Agrobacterium tumefaciens</i> (b) | Crown gall                   | 10 µg/disc   | IC          | <i>In vitro</i> | Khan <i>et al.</i> (2002)     |
| <i>Aspergillus flavus</i>            | Yellow mold                  | 400 µg/disc  | IH: 17 mm   | <i>In vitro</i> | Rahman <i>et al.</i> (2005)   |
| <i>Aspergillus fumigatus</i>         | Grain storage mold           | 25 µg/mL     | MIC         | <i>In vitro</i> | Hufford <i>et al.</i> (1980)  |
| <i>Aspergillus niger</i> ATCC 16888  | <i>Aspergillus</i> crown rot | 3.1 µg/mL    | MIC         | <i>In vitro</i> | Hufford <i>et al.</i> (1980)  |
| <i>Aspergillus niger</i>             | <i>Aspergillus</i> crown rot | 400 µg/disc  | IH: 15 mm   | <i>In vitro</i> | Rahman <i>et al.</i> (2005)   |
| <i>Aspergillus versicolor</i>        | Grain storage mold           | 400 µg/disc  | IH: 28 mm   | <i>In vitro</i> | Rahman <i>et al.</i> (2005)   |
| <i>Botrytis fabae</i>                | Gray mold                    | 38 µg/mL     | B           | <i>In situ</i>  | Hufford <i>et al.</i> (1980)  |
| <i>Cladosporium cladosporioides</i>  | <i>Hormodendrum</i> ear rot  | 2 µg; 100 µg | IC          | <i>In vitro</i> | Monteiro <i>et al.</i> (2007) |
| <i>Cladosporium sphaerospermum</i>   | <i>Cladosporium</i> rot      | 100 µg       | IC          | <i>In vitro</i> | Monteiro <i>et al.</i> (2007) |
| <i>Cryptococcus neoformans</i>       |                              | 12.5 µg/mL   | MIC         | <i>In vitro</i> | Zhang <i>et al.</i> (2001)    |
| <i>Erysiphe polygoni</i>             | Powdery mildew               | 300 µg/mL    | B           | <i>In situ</i>  | Wu (2006)                     |
| <i>Fomitopsis pinicola</i>           | Brown crumbly rot            | 2 µg/mL      | MIC         | <i>In vitro</i> | Wu (2006)                     |
| <i>Gloeophyllum trabeum</i>          | Wood rots                    | 2 µg/mL      | MIC         | <i>In vitro</i> | Wu (2006)                     |
| <i>Helminthosporium teres</i>        | Barley brown patches         | 38 µg/mL     | B           | <i>In situ</i>  | Wu (2006)                     |
| <i>Laetiporus sulphureus</i>         | Brown cubical rot            | 2 µg/mL      | MIC         | <i>In vitro</i> | Wu (2006)                     |
| <i>Lenzites betulina</i>             | Wood decay                   | 0.76 µg/mL   | MIC         | <i>In vitro</i> | Wu (2006)                     |
| <i>Phytophthora infestans</i> (p)    | Potato late blight           | 300 µg/mL    | A           | <i>In situ</i>  | Hufford <i>et al.</i> (1980)  |
| <i>Plasmopora viticola</i> (p)       | Downy mildew of grapes       | 38 µg/mL     | B           | <i>In situ</i>  | Hufford <i>et al.</i> (1980)  |
| <i>Puccinia graminia-tritici</i>     | Stem rust, cereal rust       | 300 ppm      | B           | <i>In situ</i>  | Hufford <i>et al.</i> (1980)  |
| <i>Pyricularia oryzae</i>            | Rice leaf blast              | 38 µg/mL     | B           | <i>In situ</i>  | Wu (2006)                     |
| <i>Trametes versicolor</i>           | Wood rot                     | 3.5 µg/mL    | MIC         | <i>In vitro</i> | Khan <i>et al.</i> (2002)     |
| <i>Xanthomonas campestris</i> (b)    | Leaf spots, blights          | 10 µg/disc   | IC          | <i>In vitro</i> | Khan <i>et al.</i> (2002)     |
| <i>Syncephalastrum racemosum</i>     | Black soft rot               | 3.1 µg/mL    | MIC         | <i>In vitro</i> | Hufford <i>et al.</i> (1980)  |

A, 97 to 100% disease control; B, 90 to 96% disease control; IH, inhibition halo; IC, inhibitory concentration; MIC, minimum inhibitory concentration; b, bacteria; p, protozoan.

that it likely participates in plant defense responses.

The presence and abundance of liriodenine in several Annonaceae family species has been reported (Guinaudeau *et al.*, 1994; Bentley, 2002, 2003, 2004, 2005, 2006). In particular, liriodenine is rich in *Annona diversifolia* roots of adult specimens, and is present in early germination stages in endosperm and rootlets, as well as in roots and stems of seedlings (González-Esquinca *et al.*, 2005). A seedling with two to six leaves at approximately 30 to 50 days of age has about the same amount of liriodenine as an adult plant (0.2% of the plant dry weight) (González-Esquinca, 2001; De la Cruz-Chacón and González-Esquinca, 2011). This early production indicates that it is a substance of biological significance during early development. Furthermore, during seed storage and imbibition for germination *in vitro*, seeds are often attacked by fungi that manage to complete-

ly break down the endosperm, inflicting damage on the embryo (rudimentary in Annonaceae). However, if seeds are able to germinate, the fungal population decreases as the embryo develops, a phenomenon that coincides with the emergence and subsequent increase of liriodenine. This suggests that the presence of liriodenine may inhibit the growth of fungal pathogens. To investigate a possible defense role of liriodenine, we evaluated the activity of purified liriodenine and alkaloidal extracts from *Annona diversifolia* embryo radicles in early developmental stages, seedling radicle, and roots against fungi that are phytopathogenic for this species.

## Methods and Materials

### Plant material

In September 2007, roots were collected along with 10,000 seeds of *A. diversifolia* in Delegación

de Copoya, Chiapas, Mexico. The reference specimen (352) was deposited in the Eizi Matuda Herbarium, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, México.

#### *Developmental stages evaluated*

*A. diversifolia* at five stages of early development was obtained: unimbibed seeds, day 1 of imbibition, days 2 and 10 of germination (radicles of 1 and 5 cm, respectively), and 50-day-old seedlings (with six leaves). To this end, 500 seeds were randomly selected for each assay and germinated using the technique of germination between paper (Ministerio de Agricultura, 1976). To reach the desired stage of development, seeds were placed within Conviron™ series CMP4030 plant growth chambers (Winnipeg, Canada) and maintained at 24–28 °C with 12 h of light at an intensity of 500  $\mu\text{mol}/(\text{m}^2 \text{ s})$ , achieved using white light from fluorescent lamps, and relative humidity between 65% and 75%. For each stage, embryos, radicles, and roots were carefully separated, and 10 g (fresh weight) plant material, or 2 g for embryos, were used for alkaloid extraction. Tests were conducted in quintuplicate.

#### *Total alkaloid extraction from plant organs*

Plant material was dried at room temperature. After being ground thoroughly, samples (10 g) were wetted with a saturated solution of  $\text{Na}_2\text{CO}_3$  (35 mL) and then allowed to dry for 48 h at room temperature. Alkaloids were extracted with  $\text{CHCl}_3$  (250 mL) under constant agitation for 1 h, subjected to filtration to obtain the chloroform phase, and then washed with distilled water. The chloroform extracts were extracted with 1 M HCl (125 mL), and then the aqueous phase was alkalized to pH 9.5 with a saturated  $\text{Na}_2\text{CO}_3$  solution, after which it was extracted again with  $\text{CHCl}_3$  (60 mL). Excess water was removed with anhydrous  $\text{Na}_2\text{SO}_4$ . To obtain a total alkaloid extract, the solvent was evaporated *in vacuo* at 25 °C. The alkaloid mixture gave a positive reaction with Dragendorff reagent and was subsequently analysed by high-performance liquid chromatography (HPLC). All extractions were performed in quintuplicate.

#### *Liriodenine source and identification*

Liriodenine was isolated from roots of *A. diversifolia* as previously described (De la Cruz-Chacón

and González-Esquinca, 2011). A purified sample of liriodenine was identified by Dr. Mariano Martínez Vázquez, Instituto de Química, Universidad Nacional Autónoma de México (UNAM), Cd. de México, Distrito Federal, México.

#### *Quantification of liriodenine in extracts*

Liriodenine was quantified by liquid chromatography using a Perkin Elmer HPLC system, Series NCI 200 (Norwalk, CT, USA) and a Spheri-5 RP-18 reverse phase column (100 x 4.6 mm; 5  $\mu\text{m}$  particle diameter). The mobile phase consisted of water/methanol (30:70, isocratic), and the flow rate was 1 mL/min; the column temperature was maintained at 30 °C. UV detection was conducted at 254 nm. A calibration curve was obtained by analysing a series of stock solutions containing 100, 50, 25, 6.25, 3.13, 1.56, 0.78, 0.39  $\mu\text{g}/\text{mL}$  of liriodenine. The curve was linear in this range, with an  $r$  value of 0.99 and  $r^2 = 0.98$ . The assay conditions were determined using the Perkin Elmer Turbochrom work platform. Extracts were dissolved at a concentration of 1 mg/mL, and then 20  $\mu\text{L}$  of this solution was analysed by HPLC. All liriodenine peaks were compared to its characteristic absorption bands [248 (log  $\epsilon$  4.47), 272 (log  $\epsilon$  4.41), 310 (log  $\epsilon$  3.91), and 414 nm (log  $\epsilon$  4.01)]. The absorption spectra were obtained using a Thermo Electron Corporation Genesys 10 UV spectrophotometer (Madison, WI, USA).

#### *Isolation of fungi from A. diversifolia seeds*

Ten seeds that were contaminated with fungi during the process of imbibition were selected. Fungal samples were obtained with a sterile microbiological loop, and then cultured in culture plates on BD BIOXON™ Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) (Cuatitlan Izcalli, Estado de México, México) in a RIOSSA™ (Cd. de México, Distrito Federal, México) incubator for 7–8 d at 30 °C. The most abundant colonies were selected and then used for seven consecutive reseeds in both types of culture media to isolate and purify the microorganisms. The isolated fungi were inoculated on SDA to obtain strains, which were stored in glycerol and/or liquid paraffin at 4 °C until use. *Rhizopus stolonifer* and *Aspergillus glaucus* were identified at the phytosanitary laboratory “Grupo de Estudios Moleculares aplicados a la Biología” (GeMBio), Centro de Investigación Científica de

Yucatán (CICY), Mérida, México, using morphometric keys from PDA cultures. Specimens were registered with the identification keys GeMBio NACF0010 and NACF0011. For activity assays, fungi were reseeded twice consecutively in SDA and incubated at 37 °C for intervals of 24 to 72 h before use.

#### Antifungal assays

Liriodenine antifungal activity was determined by the agar diffusion assay (Bauer *et al.*, 1966), minimum inhibitory concentration (MIC) in macrodilution (NCCLS, 1997), and glucose metabolism assay (Riesselman *et al.*, 2000; Li *et al.*, 2000). Extract activity was tested only by the glucose metabolism assay. For all three methods, we used an inoculum of  $1-5 \cdot 10^6$  CFU/mL in Sabouraud dextrose broth (SDB) (Difco BD, Sparks, MD, USA) adjusted turbidimetrically to 0.1 absorbance units at 530 nm, according to a McFarland 0.5 scale, using a Thermo Electron Genesys 10 UV spectrophotometer. All determinations were made in triplicate. For the agar diffusion assay, we used Whatman No. 2 paper discs (5 mm in diameter) impregnated with 400, 200, 100, and 50 µg of liriodenine. Tests were conducted on SDA, and inhibition halos were measured after 48 h of incubation with *R. stolonifer* and after 72 h with *A. glaucus* at 37 °C.

Determination of MIC values was performed in a final volume of 2 mL of SDB at 37 °C. Liriodenine was dissolved in ethanol (Baker™, Xalostoc, Estado de México, México) at a final content of 0.5% v/v, and then evaluated at concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.25 and 1.1 µM. The cultures were incubated for 48 h at 37 °C. MIC was determined as the lowest concentration that inhibited growth.

Inhibition of the glucose metabolism was assessed by determining the amount of glucose con-

sumed by the fungus as a source of carbon and energy after 48 h. Liriodenine was evaluated at concentrations of 100, 10, 1 µM, and extracts were evaluated at a concentration of 100 µg/mL. The assay was performed in 2 mL of SDB containing 20 mg/mL of glucose. Fungal inhibition was measured indirectly by determining the residual amount of glucose in the medium after 48 h of incubation at 37 °C. Glucose concentration was assessed by the Nelson-Somogyi method of determining reducing sugars using linear regression of a standard curve. The percentage of residual glucose for each concentration of liriodenine was calculated by comparing the glucose concentration in each assay with the glucose concentration in the culture medium, according to the following formula: % residual glucose = (assay glucose concentration/culture medium glucose concentration) · 100. The percent inhibition of glucose metabolism due to liriodenine was determined according to the following formula: % glucose inhibition =  $[1 - (100 - \% \text{ residual glucose in each concentration} / 100 - \% \text{ residual glucose in growth control})] \cdot 100$ .

#### Statistical analysis

All experimental data were expressed as means ± standard deviation of five replicates ( $n = 5$ ). Data were analysed by ANOVA, and further analysed by the Fisher's least significant difference (LSD) test when significant differences were detected. A  $P$  value < 0.05 was considered significant.

#### Results

Liriodenine inhibited the growth of both species of fungi in all assays (Table II). The inhibitory effect of liriodenine was more significant for *A. glaucus* (MIC 100 µM) than *R. stolonifer* (MIC

Table II. Liriodenine antifungal activity in different assays.

| Fungus               | Assay diffusion<br>(inhibition zone in mm)    |                        |                       |                       | Inhibition of glucose<br>metabolism (%)  |                       |                       | MIC      |         |
|----------------------|-----------------------------------------------|------------------------|-----------------------|-----------------------|------------------------------------------|-----------------------|-----------------------|----------|---------|
|                      | Tested concentration<br>[µg/disc (µmol/disc)] |                        |                       |                       | Tested concentration<br>[µmol/L (µg/mL)] |                       |                       | [µmol/L] | [µg/mL] |
|                      | 50 (0.18)                                     | 100 (0.36)             | 200 (0.73)            | 400 (1.46)            | 1 (0.27)                                 | 10 (2.75)             | 100 (27.5)            |          |         |
| <i>A. glaucus</i>    | 7 <sup>a</sup> ± 0.8                          | 11 <sup>ab</sup> ± 1.5 | 16 <sup>b</sup> ± 2.1 | 26 <sup>c</sup> ± 4.5 | 55 <sup>a</sup> ± 5.0                    | 67 <sup>b</sup> ± 3.6 | 80 <sup>c</sup> ± 6.0 | 100.0    | 27.5    |
| <i>R. stolonifer</i> | 0 ± 0.0                                       | 7 <sup>b</sup> ± 0.6   | 10 <sup>c</sup> ± 1.5 | 12 <sup>d</sup> ± 0.7 | 10 <sup>a</sup> ± 5.1                    | 35 <sup>b</sup> ± 4.7 | 55 <sup>c</sup> ± 6.8 | 200.0    | 55.1    |

Values in a row followed by different letters are significantly different ( $P \leq 0.05$ ; LSD test).



Table III. Antifungal activity of alkaloid extracts (at 100 µg/mL).

| Tested extract                     | Inhibition of glucose metabolism (%) |                          |
|------------------------------------|--------------------------------------|--------------------------|
|                                    | <i>A. glaucus</i>                    | <i>R. stolonifer</i>     |
| Embryo not imbibed                 | 0.0 <sup>a</sup> ± 0.0               | 0.0 <sup>a</sup> ± 0.0   |
| Embryo with 1 day imbibition       | 0.0 <sup>a</sup> ± 0.0               | 0.0 <sup>a</sup> ± 0.0   |
| Seedlings with radicle of 1 cm     | 29.7 <sup>b</sup> ± 1.9              | 9.2 <sup>a</sup> ± 3.1   |
| Seedlings with radicle of 5 cm     | 81.8 <sup>c</sup> ± 12.3             | 79.9 <sup>b</sup> ± 10.1 |
| Roots of seedlings with six leaves | 89.2 <sup>c</sup> ± 5.7              | 84.3 <sup>b</sup> ± 9.9  |

Values in a row followed by different letters are significantly different ( $P \leq 0.05$ ; LSD test).

200 µM) ( $P = 0.05$ ). For *A. glaucus*, the susceptible concentration of liriodenine was 50 µg in the diffusion assay ( $P = 0.0001$ ) and 1 µM in the glucose metabolism assay ( $P = 0.003$ ) while for *R. stolonifer* these concentrations were 100 µM ( $P = 0.0000$ ) and 10 µM ( $P = 0.0002$ ), respectively. For concentrations that had no visible effects, neither of the microorganisms was able to sporulate. The results of the glucose metabolism assay suggested that the effects of liriodenine are not immediate, or at least not within 48 h, since at all concentrations both fungi consumed a similar percentage of glucose.

For the alkaloid extracts of *A. diversifolia*, a concentration of 100 µg/mL was able to inhibit the *in vitro* glucose consumption of both fungi ( $P = 0.0000$ ). The inhibitory effect was slight in extracts of 1-cm radicles, whereas in extracts of 5-cm radicles and seedling roots activity increased significantly by 3- and 9-fold ( $P = 0.0000$ ;  $P = 0.0000$ ) (Table III). The activity of these ex-

tracts was similar to that of pure liriodenine in the range of 2.75–27.5 µg/mL. The liriodenine content in 5-cm radicle extracts was in this range, while in the seedling roots the quantity was two to 25 times higher ( $P = 0.0000$ ), which explains the increased activity of the latter (Table IV).

The chromatographic profiles of the extracts were poor, revealing between three and five compounds, including liriodenine, as the major components. The presence of these other compounds apparently did not have significant effects at the concentrations assayed, because if they were the major components of the extract (seeds with 1-cm radicles), the extract did not exhibit sufficient inhibitory activity. When the presence of liriodenine was more prominent in the extracts, antifungal activity increased (Tables III and IV). The bioassays supported this conclusion. Table II shows that at all concentrations tested, liriodenine was less active against *R. stolonifer* than *A. glaucus*. However, extracts from 5-cm radicles and seedling root extracts (Table III) each exhibited the same level of activity against both species. This would not be the case if the only active compound in the extracts was liriodenine. Furthermore, the chromatographic profile of the 1-cm radicle extract showed that liriodenine was present in minor quantities, yet there was still some inhibition of the two fungi, which was further evidence for one or more bioactive compounds in the extracts in addition to liriodenine.

The antifungal activity of *A. diversifolia* extracts, and of pure liriodenine at concentrations present in plant extracts, indicated the importance of this alkaloid as a component of extract activity. However, the results suggested that there is at least a second alkaloid, in lower portion than liriodenine, in seedling root extracts that may contribute to the antifungal activity.

Table IV. Liriodenine presence in alkaloid extracts of *Annona diversifolia*.

| Developmental stage            | Time [d] | Alkaloidal extract (%) | Liriodenine in extract (%) | Liriodenine content [µg/g dried tissues] | Other alkaloids (%) |
|--------------------------------|----------|------------------------|----------------------------|------------------------------------------|---------------------|
| Dry seeds                      | 0        | 0.0                    | -                          | -                                        | -                   |
| Seeds imbibed for 1 day        | 2        | 0.0                    | -                          | -                                        | -                   |
| Seedlings with radicle of 1 cm | 13       | 0.004 ± 0.00           | 2.84 ± 0.51                | 1.13 <sup>a</sup> ± 0.39                 | -                   |
| Seedlings with radicle of 5 cm | 24       | 0.34 ± 0.01            | 19.2 ± 2.28                | 125.51 <sup>b</sup> ± 22.9               | -                   |
| Seedlings with six leaves      | 50       | 0.12 ± 0.02            | 69.06 ± 4.22               | 849.04 <sup>c</sup> ± 177.58             | 10.7 ± 1.9          |

Values in a row followed by different letters are significantly different ( $P \leq 0.05$ ; LSD test).

## Discussion

Early biosynthesis of liriodenine in *A. diversifolia* is a phenomenon similar to that seen in *Glycine max*, whose 7 day-old seedlings are able to synthesize conjugated isoflavones (daidzein and genistein) before and during infection by *Phytophthora sojae*, in quantities sufficient to inhibit the phytopathogen (Graham, 1991; Lozovaya *et al.*, 2004). This is similar to several cereal species that biosynthesize hydroxamic acids as an anticipated response to attack by aphids (*Metopolophium dirhodum*, *Rhopalosiphum maidis*, *Schizaphis graminum*, and *Sitobion avenae*). Barley, rye, and wheat seeds start producing hydroxamic acids on the second day of germination (Niemeyer, 1988).

Germination and early seedling development are critical stages of plant development that allow plants to settle effectively in an environment. It appears that the presence of secondary metabolites (*i.e.* liriodenine in *A. diversifolia*) is an important strategy during this process.

The two fungal species examined in the current study are phytopathogenic for several species of the Annonaceae family. *R. stolonifer* invades the fruit pulp of *Annona muricata* to cause brown rot in the fruits, and is considered a serious post-harvest problem (Pinto *et al.*, 2005). *A. glaucus* feeds on the seed energy reserves of different cereal species (Christensen *et al.*, 1990; Lillehoj, 1992); thus, inhibition would have beneficial effects on the developing plant.

The potency of liriodenine against *R. stolonifer* and *A. glaucus* was lower than that of other fungal phytopathogens (Table II), although at the concentrations observed in the current study (MIC values of 27.5 and 55.1  $\mu\text{g/mL}$ ), this compound can inhibit the majority of such microorganisms. We propose bacterial gyrase as a probable molecular target, similar to topoisomerase II, an enzyme involved in DNA replication and transcription, which was shown by Woo *et al.* (1997, 1999) to be susceptible to liriodenine.

The amount of liriodenine in 5-cm radicles and in seedling roots was about two to 14 times higher

(125.51 and 849.04  $\mu\text{g/g}$  dried tissue, respectively) than the *in vitro* MIC for *A. glaucus*, four to 28 times higher than the MIC for *R. stolonifer*, and 90 to 300 times higher than that of other phytopathogens. In addition, according to De la Cruz-Chacón and González-Esquinca (2011), liriodenine is present during germination in endosperm at concentrations of 0.6–36.6  $\mu\text{g/g}$  tissue, and in hypocotyls and seedling stems at concentrations of 7.1–181.9  $\mu\text{g/g}$  tissue. Thus, essentially the entire developing embryo would have concentrations of liriodenine equal to that required *in vitro* to achieve partial or total inhibition of fungal growth. These observations manifest energy resource allocation to early liriodenine biosynthesis and its possible role in defense mechanisms during the initial stages of *A. diversifolia* development.

The fact that non-germinated seeds are susceptible to attack by these fungi implies that secondary metabolites detected in seeds, including acetogenins, laherradurin, rolliniastatin-2, isorolliniastatin, and cherimolin-2 (González-Esquinca, 2001; Schlie-Guzmán *et al.*, 2009; Luna-Cazáres and González-Esquinca, 2010), are not sufficient barriers to the degradation of reserve material. Laherradurin and rolliniastatin-2 are found in seeds in quantities (1000 and 30  $\mu\text{g/g}$  tissue, respectively) similar to that of liriodenine during germination. Given that *A. glaucus* and *R. stolonifer* are resistant to these compounds at such concentrations, and the fact that the presence of alkaloids is required for fungal inhibition, liriodenine, which is the most abundant of the alkaloids, most likely plays an important role in defense mechanisms in *A. diversifolia*, and perhaps also in other species as well. Furthermore, other bioactive compounds in addition to liriodenine most likely participate in these defenses.

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